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Roles for α1 Connexin in Morphogenesis of Chick Embryos Revealed Using a Novel Antisense Approach

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ABSTRACT Gap junctional communication has been implicated in embryonic development and pattern formation. The gap junction protein, α1 connexin (Cx43) is expressed in dynamic and spatially restricted patterns in the developing chick embryo and its expression correlates with many specific developmental events. High levels of expression are found in regions of budding, which leads to shaping and appears to be a necessary prelude for tissue fusions. In order to investigate the role of α1 connexin in these morphogenetic events, we developed a novel method of applying unmodified antisense deoxyoligonucleotides (ODNs) to chick embryos. The use of pluronic gel to deliver antisense ODNs has allowed us to regulate the expression of α1 connexin protein, both spatially and temporally. This "knockdown" results in some striking developmental defects that mimic some common congenital abnormalities, such as spina bifida, anencephaly, myeloschisis, limb malformation, cleft palate, failure of hematopoiesis, and cardiovascular deformity. The results imply a major role for α1 connexin communication in the integration of signaling required for pattern formation during embryonic development. This novel antisense technique may also be widely applicable. Dev. Genet. 24:33–42, 1999. © 1999 Wiley-Liss, Inc.

Key words: gap junctions; development; pattern formation; Pluronic gel; defects; connexin 43

INTRODUCTION

Gap junctions are cell membrane structures that facilitate direct cell-cell communication. A gap junction channel is formed of two hemichannels (connexons), each composed of six connexin subunits (connexins are a family of proteins, commonly named according to their molecular weight, although in this paper we refer to them by the Greek nomenclature of the two subfamilies [Bruzzone *et al.*, 1996; Risek *et al.*, 1990; Kumar and Gilula, 1996]). Gap junctional communication has been implicated in patterning and development of vertebrate embryos (reviewed by Lo, 1996) because perturbing communication or connexin expression results in developmental defects; blocking antibodies [Warner *et al.*, 1984; Fraser *et al.*, 1987; Becker *et al.*, 1992; Green *et al.*, 1994; LeClerc *et al.*, 1994; Makarenkova *et al.*, 1997], dominant negative connexins [Paul *et al.*, 1995] and knockout mice [Reaume *et al.*, 1995; Ewart *et al.*, 1997]. However, connexins are also expressed in dynamic and spatially controlled patterns in many developing organs. We have identified some of the specific events in the embryonic development of the chick that are correlated with high levels of expression of one of the gap junction proteins, α1 connexin (cx43). Using an innovative antisense deoxyoligonucleotide approach, we have transiently reduced expression of this protein in these tissues for 24–48 h. This leads to dramatic but precisely defined aberrations, including failure of outgrowth and fusion of facial primordia, limb and heart defects, and spina bifida. The tissues affected are only those that normally express high levels of α1 connexin a few h after treatment. A striking number of the defects mimic common congenital abnormalities. The results suggest that cell-cell communication through gap junctions composed of this connexin is particularly important in regions of tissue budding and fusion during embryo development.

MATERIALS AND METHODS

Antisense Application

We have used 30% Pluronic F-127 gel (BASF Corp) in phosphate-buffered saline (PBS) (molecular-grade

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Fig. 1. Penetration of FITC tagged ODN into treated tissue. Confocal microscope image of cells cells 2 h after application of pluronic gel containing FITC-DB1 ODN. The FITC tag shows that the ODN has entered the treated cells.

water) to deliver unmodified α1 connexin-specific antisense ODNs to the developing chick embryo [Simons *et al.*, 1992]. Chick embryos were incubated at 38°C and staged according to Hamilton and Hamburger stages [1951]. Eggs were windowed and the vitteline and amniotic membranes over the area to be treated were opened using fine forceps. After antisense application eggs were sealed with tape and replaced in the incubator for 48 h at which time most experiments were analysed, the exception being for the time-course analysis of α1 connexin "knockdown" and recovery.

Pluronic gel is liquid at low temperatures, 0–4°C, but sets when dropped onto the embryo at physiological temperature, remaining in place for at least 12 h. The rapid setting of the gel as it warms necessitates swift application and use of instruments that are kept on ice in order to prolong the working period. By varying the size of the droplet of gel, one can accurately target different areas or sizes of tissue. Small droplets of around 5 μl would cover a single limb bud or one side of the face, molding around that part of the embryo as the gel rapidly sets. Larger droplets can cover larger areas of tissue and can be run along the whole spinal cord in early embyros. The gel has the additional advantage of being a mild surfactant and this, used either alone or in conjunction with dimethylsulfoxide (DMSO), appeared to markedly expedite ODN penetration into cells. Addition of an FITC tag to DB1 ODN, viewed using confocal laser scanning microscopy, demonstrated intracellular penetration of the probes (Fig. 1). Sequences of deoxyoligonucleotides used are shown in Table 1. All ODNs were applied at 0.5–1.0 μM final concentration after dose-dependent analysis during preliminary experiments covering a range of concentrations of 0.05–50 μM. General toxicity effects only became apparent with ODN concentrations of >10 μM. ODN gel mixtures were prepared from concentrated stock solutions stored at −80°C.

Antisense Sequences

ODNs were selected on the basis of being specific for connexin α1 and of not forming homodimers, stem loops, or hairpins. Preliminary screens of potential ODNs showed that some sequences were either toxic or did not reduce α1 protein expression, or both. DB1 was designed to work as a mouse antisense sequence to the α1 connexin gene (sequence is now proprietary). It has four mismatches with chick α1 connexin sequence but is still effective in reducing chick α1 protein. CG1 is complementary to chick α1 connexin. The efficacy of this probe was improved with 1% DMSO added to the gel. DMSO had no added effect on other antisense ODN or control results (Table 1).

Controls. DB1(Chick) is the chick α1 connexin equivalent of DB1 matching chick α1 connexin. Analysis. however, indicates a high probability of forming stem loop structures ($G = -7.0$ kcal/mol, loop $T_m = 92°$) and homodimerization ($T_m = 1.5°$) and therefore acts as a control sequence. It has been reported that some sense oligonucleotides can form stable DNA triplets [Neckers *et al.*, 1993], inhibiting transcription. However, this was not apparent with DB1(sense). Random control sequences were also used. Analysis showed that these had no stable secondary structure ($G = 1.4$ kcal/mol) and homodimerization would be unstable. An additional control applying equal concentration mixture of DB1 and DB1(sense) gave background levels of defects.

Monitoring of Protein Knockdown

Immunohistochemical localization of α1 connexin gap junction protein at cell-cell interfaces provides a direct measurement of the antisense effect. Anti-peptide α1 connexin-specific antibody probes were used to stain whole-mount embryos and the connexin distribution was analysed using confocal laser scanning microscopy according to established procedures [Green *et al.*, 1994; Becker *et al.*, 1995]. Control labeling for two other connexins expressed in the developing chick embryo (connexins β1 [Cx32] and β2 [Cx26]) was similarly carried out, also using sequence-specific antibodies [Becker *et al.*, 1995]. The expression pattern of some genes, *Msx-1*, *Fgf-4*, and *Shh*, which are known to be involved in patterning was investigated using in situ hybridization according to the well-established methods in our laboratories [Nieto *et al.*, 1996].

RESULTS

Reduction of α1 Connexin Expression

Previous studies and our present results show that α1 connexin is widely expressed during chick embryonic development, showing spatial and temporal peaks of expression which correlate with specific developmental events such as limb outgrowth, fusion of the neural

TABLE 1. Effect on Limb Development of ODN Application Between Stages 8 and 14 of Chick Embryo Development

Antisense deoxyoligonucleotides to α1 connexin	Efficacy[a]	
DB1[b]	28% (n = 123)	
CG1[b]	19% (n = 72)	1% DMSO 40% (n = 44)
Control deoxyoligonucleotides		
DB1[b] (sense)	6.7% (n = 59)	
DB1[b] (chick)	6% (n = 18)	
Random 30 mers CV3 and RC2	17% (n = 54)	1% DMSO 20% (n = 15)
Gel only	7.8% (n = 64)	1% DMSO 0% (n = 5)

DMSO, dimethylsulfoxide.
[a]Efficacy expressed as percentage of embryos with gross limb patterning defects (truncation or splitting) after antisense application at stages 8–14. Limb truncations were compared by eye with the control side and minor differences in length of limb may have been missed. Embryos with multiple limb defects have been counted as one. Only surviving embryos are included; there was no significant difference in survival between ODNs.
[b]Sequence is now proprietary.

tube and fusion of the face and palate [Allen *et al.*, 1990; Minkoff *et al.*, 1991; Green *et al.*, 1994]. Using Pluronic F-127 gel to deliver unmodified α1 connexin-specific antisense ODNs to the developing chick embryo, one can interfere with protein expression at chosen time points and target the antisense treatment to specific regions of a chick embryo. A droplet of gel containing the antisense at a relatively low concentration could be placed precisely onto specific tissues or areas of an individual embryos or larger drops used to cover wider areas. The gel sets and remains in place for at least 12 h; thus, a sustained low dose of antisense is maintained in this region. Our antisense applications were targeted and timed to block junction formation prior to the periods of elevated expression in the limb, neural tube, and face. These times were chosen to optimize the effects of the antisense by reducing the expression of new protein, rather than being dependent on the turnover of protein already in the membranes of the cells of the target tissue. Both DB1 and CG1 ODNs reduced expression of α1 connexin protein. In a DB1 time series, this occurred within 2 h in the neural tube and limb bud and was dramatic within 4–8 h, persisting at 18–24 h and 48 h in some tissues (Fig. 2). Some variation in tissue types was noted. For instance, "knockdown" in somites took 8–16 h to achieve; recovery was also longer indicating a slower turnover rate of α1 connexin in this tissue compared with the neural tube and limb bud. No downregulation of α1 connexin protein was evident in any of the controls used. Two other members of the connexin family expressed in the chick embryo, β1 connexin and β2 connexin, were unaffected by the α1 connexin-specific antisense ODN (Fig. 2). However, after "knockdown" of α1 connexin protein, developmental defects are often seen in areas that normally express high levels of the protein.

Several parallel controls were run with all of the experiments. These included; DB1 sense, DB1 antisense and DB1 sense combined, DB1 chick (which forms stem loop structures with itself), random ODNs CV3 aand RC2, Pluronic gel alone, Pluronic gel with DMSO, and PBS alone). None of the controls had a noticeable effect on α1 connexin protein expression (Fig. 2). Some developing tissues seemed to be more sensitive to treatment than others. Treatment of the eye only produced changes in growth with antisense and not any of the controls, whereas treatment of the limb was more sensitive, and a low level (7%; n = 141) of truncated limbs could be produced with the controls, compared with 28% (n = 239) with antisense ODNs. Random ODNs gave a slightly higher incidence of truncations (17%; n = 54), which may reflect a mild toxicity from this probe causing growth retardation without reducing connexin expression. The percentage of defects with this probe was not significantly affected by the addition of 1% DMSO to the gel (20%; n = 15). Addition of 1% DMSO to the pluronic gel alone gave rise to no defects, although n numbers were low (n = 5). Only one incidence of limb division was found in all the controls; it is possible that this may have resulted from mechanical damage. No limb defects were produced from the addition of PBS alone (n = 18).

Consequences of α1 Connexin Knockdown

Limb buds. Reduction of α1 connexin protein expression in the limb bud after specific antisense ODN treatment often resulted in one of two striking defects—truncated or divided limb buds (Figs. 3, 4, Table 1); 28% (n = 239) of chick embryos treated antisense ODNs (DB1 and CG1), between stages 8–14 (well before limbs develop) had these limb defects. In comparison, only 9.7% of all controls (n = 195) showed limb defects. Limb truncations were the most common defect seen and could be produced with antisense ODN applications between stages 7–14. Divided limb buds were less frequent but were maximally produced with antisense ODN applications between stages 11–14. Only one divided limb bud was found among controls and may well have been produced by mechanical damage. In the case of divided limb buds, the two parts of the bud were clearly not identical indicating that the limb fields were split, rather than duplicated. Support for this notion comes from in situ hybridization studies that showed that *Shh* was expressed only in the most posterior

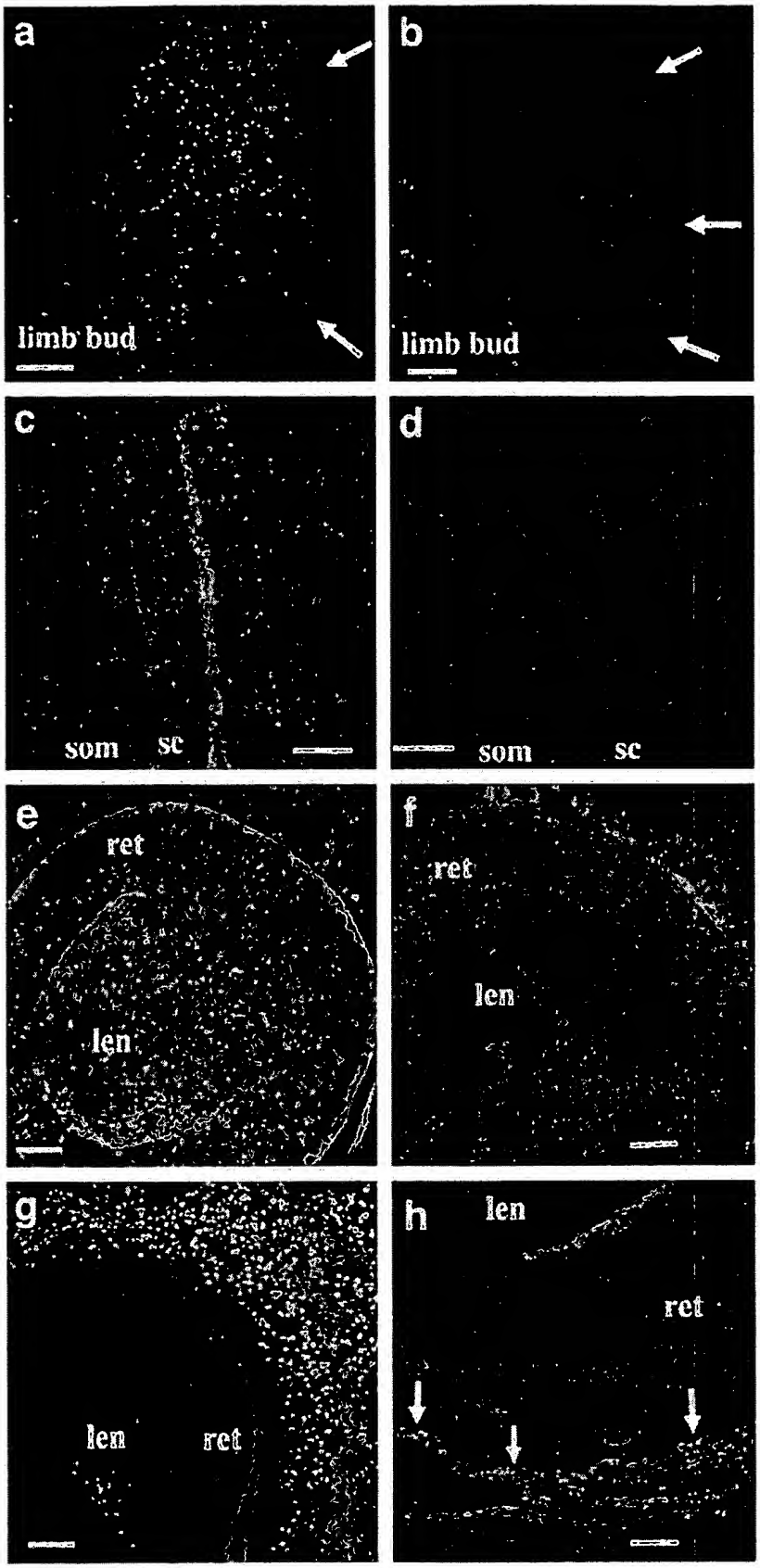

Fig. 2.

outgrowth in divided limbs and not in both as would be expected if the bud had been duplicated (Fig. 4). Expression of a homeobox gene *Msx-1* in the mesenchyme at the tip of the limb bud depends upon apical ridge signaling and treatment of embryos with antisense ODNs to α1 connexin, along the spinal cord and flank, abolished *Msx-1* expression, as well as the connexin expression (Fig. 4).

Face and palate. Outgrowth and fusions between facial primordia were also particularly sensitive to α1 connexin protein "knockdown." During chick facial development, primordia grow out and fuse in regions of high epithelial and mesenchymal α1 connexin expression [Minkoff *et al.*, 1991]. α1 connexin-specific antisense ODN application to the head region induced defects in 52% (n = 53) of embryos treated at stages 8–14. In affected embryos, a reduction in outgrowth of the maxillary primordium combined with a reduction in depth of the nasal pit was apparent. Merging of maxillary and mandibular primordia at the corners of the mouth, where α1 connexin expression is normally intense, was also affected. Embryos treated at stages 12–14 on the right hand side of the head often exhibited an uneven outgrowth of primordia on the two sides of the face. Treatments of one side of the head with α1 connexin antisense ODNs reduced the expression of this protein in the facial primordia and in the retina (Fig. 2). This resulted in a marked retardation or growth of the treated eye on the treated side in 28% (n = 53) of embryos. No significant difference in eye sizes were seen with any of the controls.

Neural tube. Another important fusion process characterized by high levels of α1 connexin expression is neural tube fusion. Fusion here also occurs in zones of high α1 connexin expression in the upper neural folds (Fig. 2). This zone of high α1 connexin expression moves down the body column in parallel with tube fusion and is affected by α1 connexin-specific antisense ODN treatment. Application of α1 connexin-specific antisense ODN to early neurulating embryos prevents fusion anteriorly (anencephaly); anterior tube fusion is not affected at later stages of application. Application of the ODN after neural tube fusion has begun (stage 8) results in posterior neuropore closure defects (spina bifida) (Figs. 3, 4) with upper head and lower tail regions of the tube formed as normal. By stage 13, neural tube closure is down to somite 19 and begins to approach the transition between primary and secondary neurulation which is marked by somite 27. Antisense ODN application between stages stages 8–14 resulted in 30% of embryos with neural tube defects (n = 53) compared with 5% (n = 40) of embryos with defects with sense ODNs. Application of antisense ODN to the lower abdomen prior to these later events in tube fusion (stages 13–16) results in failure of secondary neurulation but has no effect on primary neurulation. This later failure can be accompanied by tail truncation, and leg deletions or divisions whilst the upper portion of the embryos appears normal. As in the limb, *Msx-1* expression is tightly correlated with the α1 connexin expression in the upper regions of the neural tube. Treatment with the α1 connexin antisense ODNs reduces *Msx-1* expression in the limb (see above); we also found a marked reduction in *Msx-1* transcript levels in the neural tube (Fig. 4).

Blood vascular system. The other main region of the embryo that is affected by antisense treatment is the vascular system. Grossly defective hearts were apparent in 21% of embryos treated with the antisense ODNs over the head and mid-trunk region between stages 8–14 (n = 73), whereas sense ODNs produced only 7% (n = 40) when embryos were treated at these stages. The hearts were most commonly enlarged or protruding and often lacked the normal smooth appearance and showed small defined bulges. Occasionally hearts were also looped inappropriately. These heart defects were often accompanied by incomplete body closure.

Blood formation anomalies, notably colorless blood, indicating globin gene or erythropoietic perturbation, occurred in 26% (n = 53) of embryos treated with antisense ODNs in head and mid-trunk and 30% (n = 50) of embryos treated in mid trunk region alone at stages 8–14 and 7–14, respectively. By contrast, embryos treated with sense ODNs at stages 8–14 showed only 5% (n = 40) of blood defects. Embryos showing blood system defects rarely survived beyond stage 26. These anomalies were significantly reduced when antisense was applied to later stage embryos when migration of intraembryonic precursor hematopoietic foci from near the dorsal aorta of the embryo to yolk sac blood islands has already taken place [Cormier, 1993; Deiterlen-Lievre and Martin, 1981].

Fig. 2. Connexin expression in chick embryos after sense and antisense treatment. Immunohistochemical localization of gap junction proteins in chick embryonic tissues after α1 connexin-specific antisense oligonucleotide or control oligonucleotide applications. Confocal laser scanning microscopy images of chick limb bud whole mounts show high levels of α1 connexin expression in the mesenchyme at stage 20–21 of a sense control embryo treated 18 h before labeling (a), whereas embryos treated with connexin43-specific antisense (b) show little or no α1 connexin protein labeling. *Arrows*, edge of limb bud. The neural tube of the chick has high ectodermal α1 connexin expression around the point of fusion in normal and sense control treated embryos (c), reduced to very low levels in animals treated with antisense oligonucleotides 18 h before antibody labeling (d) [note the poor fusion of the cord]. Reduction in α1 connexin expression is specific, as shown by immunohistochemical labeling of developing eyes in stage 18–19 chick embryos ODN treated over the head region at stage 11–12. α1 connexin labeling is high in the retina (ret) and newly formed lens (len) of control treated embryos (e). α1 connexin-specific antisense ODN treated embryos show a major reduction in α1 connexin protein labeling in the retina and lens (f), but β1 connexin (g) and β2 connexin (h, *arrows*), levels are the same as in controls for these connexin types (expressed in the mesenchyme and epithelium, respectively, at this stage). sc, spinal cord; som, somite; ret, retina; len, lens; mes, mesenchyme. Scale bars: a–d, 50 μm; e–g, 25 μm.

DISCUSSION

We have developed a novel method for delivering antisense deoxyoligonucleotides (ODNs) to developing chick embryos using pluronic gel. Antisense ODNs have considerable potential as agents for the specific manipulation of gene expression [for review, see Stein *et al.*, 1992; Wagner, 1994]. However, unmodified phosphodiester oligomers (PO) typically have an intracellular half-life of only 20 min, owing to intracellular nuclease degradation [Wagner, 1994], and phosphorothioate and methylphosphonate ODNs are often used, as they are readily available and nuclease resistant. In general, though, these have a weaker affinity than that of PO ODNs, can be less efficient at entering the cells, and can cause nonspecific inhibition by binding to essential proteins [Milligan *et al.*, 1993; Wagner, 1994]. Our approach overcomes the effect of intracellular nuclease activity by providing, for a period, a constant source of ODNs from the gel, thereby reducing artifacts that can occur at higher dose levels, including nonspecific imperfectly matched mRNA binding [Woolf, 1992; Boiziau, 1994] and accumulation of nucleotide and nucleoside breakdown products that can affect cell proliferation and differentiation [Wagner, 1994]. FITC tagging of the ODNs has demonstrated their entry into the treated tissues and immunostaining and confocal microscopy showed a reduction in α1 connexin protein compared with controls in 4–8 h, which remained "knocked down" for 24–48 h (depending on tissue type). By blocking translation, rather than transcription, we appear to have eliminated compensation by other homologous connexins and have been able to demonstrate roles for α1 connexin that could not be detected in transgenic gene knockout models. The Pluronic gel–antisense oligonucleotide approach may be generally applicable to other developmental questions in chick embryos, provided that attentionis paid to the antisense ODN design and appropriate controls detailed by Wagner [1994].

Limb defects. Limb defects were frequently produced by antisense treatment and consisted of either truncated limbs or divided limbs. The truncated limb phenotype is consistent with previous experiments that showed a correlation between α1 connexin expression in limb mesenchyme and subsequent limb outgrowth [Green *et al.*, 1994]. α1 connexin expression in the mesenchyme is enhanced by grafting a second apical ridge to a limb bud, and a second outgrowth is induced, while removal of the apical ridge leads to loss of α1 connexin expression in the mesenchyme and to limb truncation [Green *et al.*, 1994; Makarenkova *et al.*, 1997]. Antisense treatment could also be directly affecting apical ectodermal ridge signaling. α1 connexin expression is detectable in the presumptive limb apical ectodermal ridge at stage 17 of development. This is approximately 6 h after the stage at which application of the antisense ODN leads to a peak incidence of truncations. Interestingly the legless mutant mouse also shows deficiencies in α1 connexin expression in its developing limb buds compared to normal mouse limbs (Meyer *et al.*, 1997; Bell *et al.*, 1998).

The divided limbs resemble those produced by ectopic expression of *Radical Fringe*, in which apical ridge development is disrupted [Rodriguez-Esteban *et al.*, 1997; Laufer *et al.*, 1997]. Thus, α1 connexin antisense treatment could have produced its effect by local lesions in the apical ridge and this would point to a role for α1 connexin in ridge assembly.

Divided buds can also be obtained when a polarising region is grafted to the apex of a wing. It is now known that *Shh* is expressed in the polarising region of the limb bud and is involved in its anteroposterior patterning. However, *Shh* is not expressed in the anterior outgrowth of the divided limbs that result from antisense treatment, supporting the idea that these divided buds result primarily from interruption of ridge formation, rather than from ectopic polarizing activity. Taken together, all these results show that expression of α1 connexin gap junctional protein in tissues at the tip of the developing chick limb is necessary for proper limb bud outgrowth [Green *et al.*, 1994]. Current experiments in our laboratories are investigating the interrelationships between connexin expression and that of other signaling molecules involved in limb bud patterning.

Face and palate. Facial defects produced by antisense treatment include a reduced outgrowth of maxillary primordium combined with a reduction in depth of the nasal pit and alterations of the merging of maxillary and mandibular primordia at the corners of the mouth. α1 connexin expression is normally high in all these regions in the associated epithelial and mesenchymal tissues [Minkoff *et al.*, 1991]. Similar facial defects have been produced previously with retinoic acid treatment. Retinoic acid is known to affect cell-cell communication through gap junctions in other systems [Mehta and Lowenstein, 1989; Mehta *et al.*, 1991]. When retinoic acid or the retinoic acid analogue TTNPB [(E)-4–2-(5,6,7,8-tetramethyl-2-naphaenyl)-1-propenyl acid] was applied to the developing chick face, primary palate formation is defective [Wedden *et al.*, 1986]; this is associated with a reduction of α1 connexin expression [McGonnell *et al.*, submitted for publication]. This, taken together with our present results, suggests that direct cell-cell communication through α1 connexin is necessary for fusion events in facial development. Since fusion depends on proper outgrowth of adjacent facial primordia, the underlying mechanisms that produce these facial defects appear to be similar to those that

Fig. 3. Developmental defects resulting from α1 connexin knockdown. Defects resulting from α1 connexin knockdown, using α1 connexin-specific antisense ODNs, after antisense application to the mid-region at stage 8–11. a: Scanning electron micrograph shown in neural tube closure in the mid-region has failed, leading to spina bifida. b: A divided wing has formed. The divided limb bud does not appear to consist of two identical halves, as the anterior "bud" has formed an apical ectodermal ridge, whereas the posterior one has not.

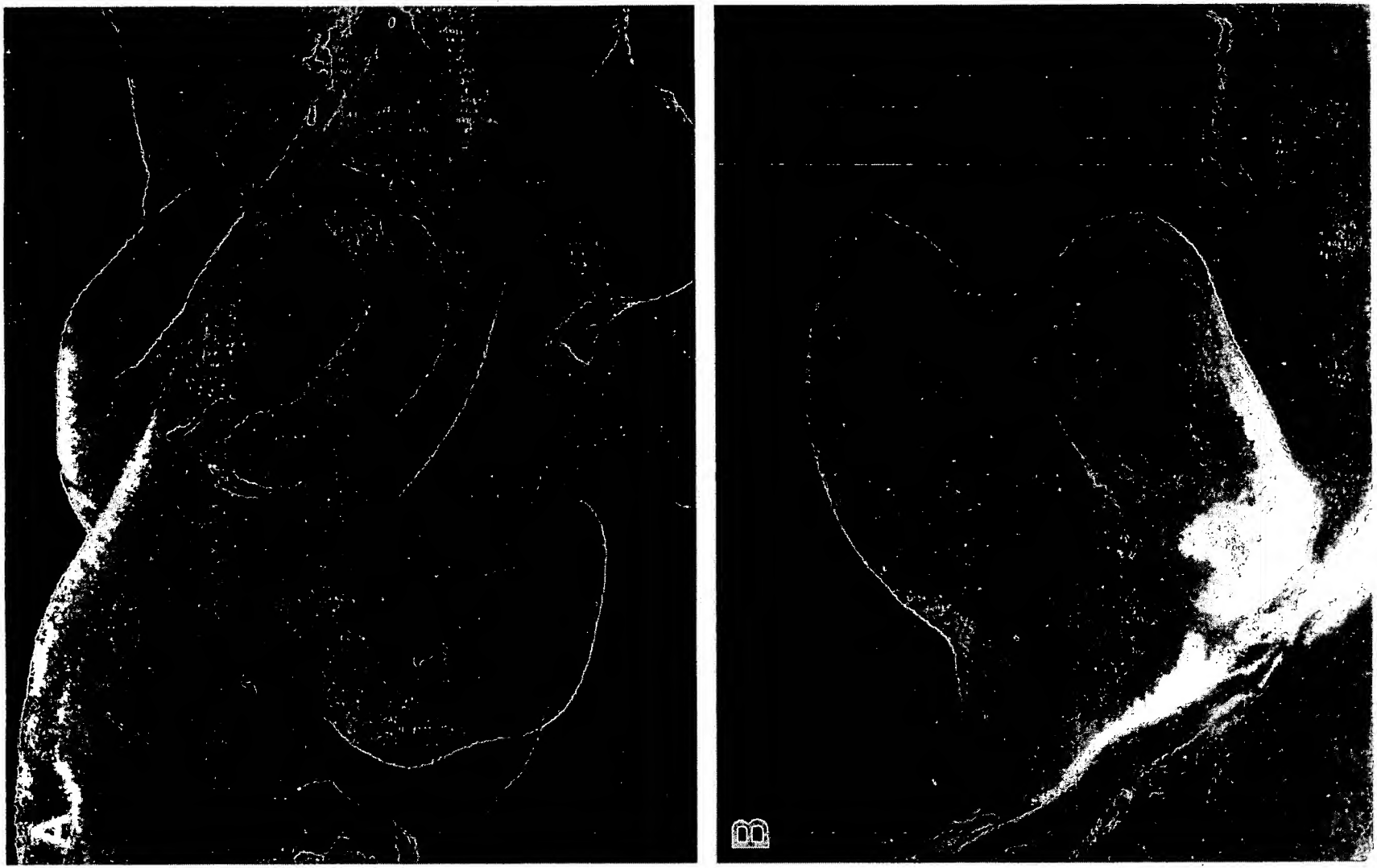

Fig. 3.

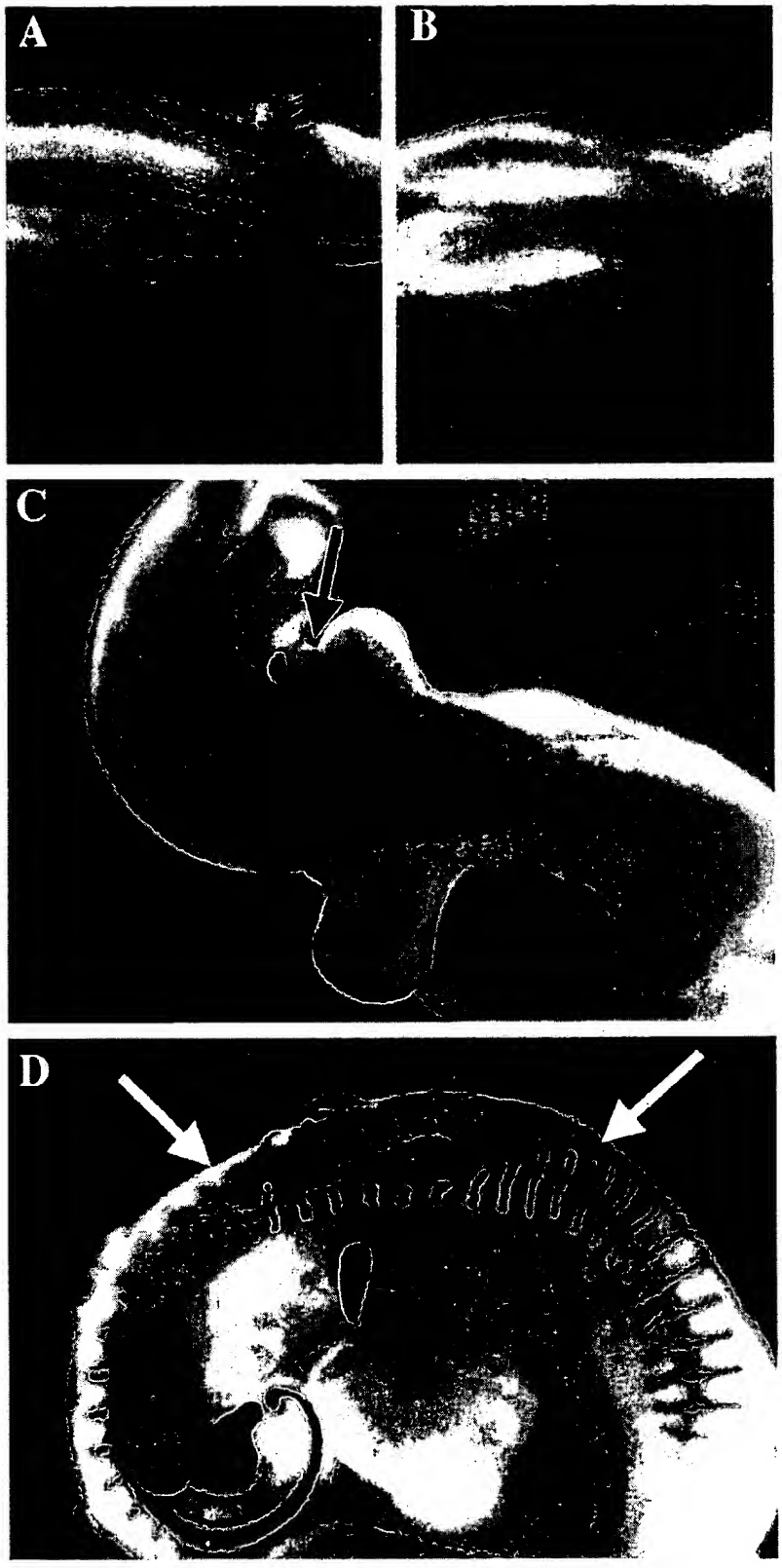

Fig. 4.

produce limb defects. Indeed, retinoic acid has previously been shown to induce similar limb defects to what we see with antisense knockdown of α1 connexin, duplications, and truncations. Previous studies have shown, independently, that retinoic acid application can reduce both α1 connexin expression and *Msx-1* expression [Wedden *et al.*, 1986; Mehta and Lowenstein, 1989; Mehta *et al.*, 1991; Yokouchi *et al.*, 1991]. While our current results show that their expressions are tightly correlated in the developing embryo and are clearly interdependent, we are unable to demonstrate their order in the cascade of gene expression involved in patterning of the face and palate or limb bud.

Neural tube. Treatment with the α1 connexin antisense ODNs reduces the α1 connexin protein expression and often results in failure of neural tube fusion. The point of fusion in the developing neural tube is a zone of high α1 connexin expression. α1 connexin is also expressed strongly in the neural crest, which leaves the neural tube around the point of fusion [Lo *et al.*, 1997]. The spatiotemporal control of the treatment and knockdown has to be very precise to produce an effect suggesting a role for communication at the point of closure. Similar neural tube defects have recently been reported in transgenic mice that overexpress α1 connexin [Ewart *et al.*, 1997] implying that an appropriate level of communication through α1 connexin is essential during embryonic development.

Blood vascular system. Defects in the development of the hematopoietic system included a lack of globin in the blood cells and also heart defects. Blocking α1 connexin translation does not appear to target a specific developmental step in the hematopoietic system, but rather seems to perturb an ongoing role for direct cell-cell communication in hematopoietic regulation. This finding is consistent with previous studies, which demonstrated that treatments that result in upregulated hematopoiesis in mice (end cell numbers) cause a 100-fold increase in gap junction numbers in bone marrow [Rosendaal *et al.*, 1994]. Cells in long-term bone marrow cultures are also extensively coupled by α1 connexin [Rosendaal *et al.*, 1991], again indicating that α1 connexin is involved in the regulation of mammalian hematopoietic stem cell populations.

Heart defects leading to neonatal death have previously been described in α1 connexin knockout mice [Reaume *et al.*, 1995]. Defects we observed in the developing heart were associated with size and shape, often being swollen or bulbous and containing very large, uneven cells on the surface, giving a raspberry-like appearance. However, the chick heart contains very little α1 connexin at these stages of development. It possible that these cardiac developmental defects could arise as a result of perturbation of migration of neural crest cells, which express α1 connexin, [Lo *et al.*, 1997], rather than by a direct effect on the heart itself. Investigations are currently under way that aim to determine this possibility.

CONCLUSIONS

We have shown that we are able to regulate specifically α1 connexin expression with a high degree of spatial and temporal resolution. We have demonstrated that, in the absence of α1 connexin expression, outgrowth and fusion events in limb, face, and neural tube are inhibited; defects were also produced in the cardiovascular system. A striking number of these defects mimic common congenital abnormalities, including anencephaly, spina bifida, myeloschisis, limb malformation, cleft palate, failure of hematopoiesis, and cardiovascular deformity. Taken together, these results suggest that communication through α1 connexin plays an important role, interacting with other signaling mechanisms during embryonic development and pattern formation. Interestingly, there is a strong correlation between *Msx-1* expression and α1 connexin expression in limb, face and palate and neural tube and we found a marked reduction of *Msx-1* both in limbs and in neural tube after antisense ODN α1 connexin knockdown. These results suggest that expression of α1 connexin is necessary to maintain *Msx-1* expression in several different regions of the embryo. In addition, this novel technique, which allows us to regulate protein expression both spatially and temporally, may be widely applicable.

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Fig. 4. Affects of α1 connexin knockdown on the expression of genes involved in patterning. Gene expression in embryos after α1 connexin-specific antisense application along the length of the spinal cord and flanks at stages 11–14 and processing for in situ hybridization as in Nieto *et al.*, 1996. *Msx-1* expression is tightly correlated with that of α1 connexin in the limb buds, neural folds, and face and palate. Normal expression of *Msx-1* in the fusing neural folds and the hindlimb buds is seen in an embryo 24 h after control sense treatment (**a**), whereas *Msx-1* is dramatically downregulated after the application of α1 connexin-specific antisense ODNs (**b**). Note that all in situ hybridization development was carried out in the presence of control tissues in order to determine the appropriate and consistent level of color development. **c**: A duplicated left wing bud; leg bud development (which occurs subsequently and lower down the animal from the site of antisense application); the right wing bud appears normal. The in situ probes used are *Fgf-4* and *Shh*. *Shh* is expressed in the polarizing region of the limb (posterior) and can be seen to be expressed only in the most posterior of the divided limb buds (*arrowed*), indicating a splitting of the limb field, rather than a true duplication. *Fgf-4* expression demarcates Von Ebners fissure that separates the somites. **d**: A case of spina bifida (*arrowed*) showing shortening and, in some cases, bifurcation of somites.

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